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Award Number: DAMD17-99-1-9499

TITLE: Cell motility and invasiveness of neurotibromin-deficient neural crest cells and malignant triton tumor lines

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REPORT DATE: October 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2002	Annual (1 Oct 2001 - 30 Sep 2002)

4. TITLE AND SUBTITLE
Cell motility and invasiveness of neurotibromin-deficient neural crest cells and malignant triton tumor lines

5. FUNDING NUMBER
DAMD17-99-1-9499

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Our purpose is to examine the role of the NF1 gene product, neurofibromin, in modulating the migratory and invasive properties of neural crest cells (NCC) and neural crest-derived sarcoma cells. As a negative regulator of Ras signaling, neurofibromin may influence the responses of NC-derived cells to growth factors and extracellular matrix (ECM) molecules that affect motility. We use embryonic NCC and NC-derived sarcoma lines isolated from cisNf1;p53 mice to compare integrin ECM receptor expression patterns, ECM adhesion preferences, migration on ECM substrata, invasion through ECM barriers, and dispersal along NCC pathways in vivo for wild-type and neurofibromin-deficient cells. In the past year, we have completed studies on the invasiveness of branchial arch neural crest cells isolated from Nf1+/+, +/-, and -/- mouse embryos, and developed methods to examine invasiveness of neural crest-derived cells in the trigeminal ganglion. In addition, we characterized the effects of TGFβ on the invasiveness and neural crest phenotype of cisNf1;p53 sarcoma cells. Our studies address two important questions: 1) what molecules control the migration and localization of NCC in the embryo? 2) which growth factor signaling pathways affect the invasiveness of NC-derived sarcoma cells?

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
neurofibromatosis type 1; neural crest cells; cell motility and Migration; extracellular matrix receptors; MTT; neurofibromin

15. NUMBER OF PAGES
22

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The neural crest of vertebrate embryos is a migratory population of stem cells that give rise to sensory and autonomic neurons, Schwann cells, melanocytes, and smooth muscle cells in the outflow tract of the heart (Weston, 1991). Neurofibromatosis type 1 (NF1) is in large part a disorder of neural crest-derivatives, and the neurofibromin protein regulates the responses of neural crest-derived neurons, Schwann cells, and melanocytes to a variety of environmental cues. Mice harboring targeted null muatations in the Nf1 and Trp53 tumor suppressor genes in the cis configuration spontaneously develop malignant soft tissue sarcomas, including malignant peripheral nerve sheath tumors and malignant Triton tumors (Cichowski et al., 1999; Vogel et al., 1999). The objectives of the proposed research are to 1) analyze the effects of Nf1 gene dosage on the migratory and invasive properties of primary neural crest cells isolated from E9-E11.5 mouse embryos, and 2) to characterize the contributions of TGFβ and Ras signaling to the invasive and motile properties of neural crest-derived cisNf1;p53 sarcoma cells. Over the past year, we have begun to characterize abnormalities in cranial neural crest distribution and trigeminal axon patterning in Nf1-/- mouse embryos (Task 1), and completed studies on the invasiveness of branchial arch mesenchymal cells (Task 2). In addition, we have characterized effects of TGFβ on cisNf1;p53 sarcoma cell invasiveness and expression of neuronal markers (Task 3).

BODY

Task 1: Compare neural crest cell (NCC) dispersal in wild-type and Nf1-/- mouse embryos

Wehrle-Haller and Weston (1995) assessed melanocyte precursor migration on the lateral pathway in Steel mutant mouse embryos, which lack the ligand for the c-kit receptor tyrosine kinase (mast cell growth factor or stem cell factor). They found that melanocyte precursors failed to enter the lateral pathway in Steel null embryos, and ultimately disappeared from the migration staging area. In Steel-dickie mutant embryos, which produce only the soluble form of stem cell factor, melanocyte precursors migrate on the lateral pathway, but fail to survive. Because Nf1-/- peripheral neurons survive in the absence of growth factors (Vogel et al., 1995), and because Nf1 haploinsufficiency partially rescues the coat-color deficiency in c-kit mutant mice (Ingram et al., 2000), Wehrle-Haller and colleagues combined Nf1 and Steel mutations in order to assess the migratory behavior of melanocyte precursors in the absence of stem cell factor. They reported that melanocyte precursors migrate normally in Nf1-/- embryos, and are independent of stem cell factor for survival; however, in the absence of stem cell factor, Nf1-/- melanocyte precursors remain in the migration staging area and do not disperse (Wehrle-Haller et al., 2001).

Based on these results and our own work with neurofibromin-deficient neural crest populations, we predict that neuronal precursors will persist on the lateral migration pathway of Nf1-/- mouse embryos. Since we know that dorsal root and sympathetic ganglia form in the correct locations in Nf1 mutant mouse embryos, we do not expect neuronal precursor migration along the medial pathway to be perturbed in the absence of neurofibromin. However, our preliminary results indicate that neural crest and/or placodal cell migration into the trigeminal ganglion is abnormal, and that initial axon outgrowth from trigeminal sensory neurons into ophthalmic, maxillary, and mandibular regions is perturbed in Nf1-/- embryos (C. Geyer and K. Vogel, unpublished observations). Over the next year, we will continue to examine neuronal precursor migration and distribution in both the trunk and cranial regions of Nf1-/- embryos between E8.5 and E12, with particular attention to rhombomere 1 and 2 neural crest cells that migrate into the trigeminal ganglion, and maxillary and mandibular arches (Couly et al., 2002). To facilitate these experiments, I have hired Mr. Rene Garza as a research assistant to work on sectioning and immunostaining mouse embryos; Mr. Garza will also assist with the experiments outlined in Tasks 2 and 3.

Task 2: Compare expression of integrin ECM receptors and motile characteristics for wild-type and Nf1-/- mouse NCC

Following promising preliminary experiments (reported last year), we continued to focus our efforts on analyzing the invasive properties of cranial neural crest cells isolated from wild-type and Nf1-/- mouse embryos. **Figures 1 and 2** each summarizes results obtained from 2 or 3 separate experiments, with NCC isolated from at least 3 different embryos of each genotype. At embryonic day 10 (E10), only the mandibular arch is distinct enough to be microdissected; we observed no significant differences in invasiveness through fibronectin matrices between the three genotypes. However, E10 Nf1-/- mandibular arch NCC exhibit increased invasiveness through laminin, when compared to NCC isolated from stage-matched Nf1+/- and +/+ littermates (**Figure 1**). By E11.5, maxillary and mandibular arches can be distinguished and microdissected separately. **Figure 2** shows that both maxillary and mandibular Nf1-/- NCC exhibit increased invasiveness through fibronectin and laminin matrices, when compared to first arch NCC isolated from Nf1+/- and +/+ littermates.

To address the concern that maxillary and mandibular arch cultures do not represent pure populations of cranial NCC and may also contain paraxial and angiogenic mesodermal cells, we developed microdissection techniques to isolate NC-derived cells from E10-E13 trigeminal ganglia. Sensory neurons in the trigeminal ganglion are derived from both neural crest and epidermal placode; non-neuronal cells in the ganglia arise solely from neural crest (D'Amico-Martel and Noden, 1983). Moreover, the non-neuronal cells of the trigeminal ganglion arise from the same hindbrain region as do the NC-derived mesenchymal cells of the maxillary and mandibular arches. Following microdissection of trigeminal ganglia, we expand the non-neuronal cell population in Neurobasal medium + 5% fetal calf serum, and then challenge the cells with our standard transwell invasiveness assays. **Figure 3** shows that trigeminal ganglion non-neuronal cells isolated from E11.5 Nf1-/- mouse embryos are more invasive through both fibronectin and laminin matrices than are their Nf1+/- and +/+ counterparts. Although neurons survive in the absence of nerve growth factor (NGF) in cultures of Nf1-/-, but not Nf1+/- or +/+, trigeminal ganglia (Vogel et al., 1995), the morphology of the cells that invade through the matrices is inconsistent with a neuronal phenotype. Nevertheless, we can address this issue of differential invasiveness by maintaining the trigeminal ganglia in the presence of NGF during expansion of the non-neuronal population. Additionally, we can immunostain the cells on the membrane for markers of neurons, Schwann cells, melanocytes, and their precursors.

Another concern for these experiments is that Nf1-/- NCC may proliferate more extensively during the 20-hr invasiveness assay period, and thus affect the number of cells counted, even though the same number of cells is plated on top of the matrix initially for each genotype. Both Nf1+/- mast cells (Ingram et al., 2001) and astrocytes (Gutmann et al., 1999) exhibit increased proliferation; however, we observed no significant differences in invasiveness between Nf1+/+ and Nf1+/- cranial neural crest cells. To be counted in the transwell assay, a NCC would have to migrate through the matrix, and then undergo mitosis on the transwell membrane in the presence of a serum concentration between 0 and 2%. To address this, we have begun to compare the percentages of cells entering S-phase by measuring bromodeoxyuridine incorporation in 2% serum during a 20-hr pulse, for Nf1-/-, +/-, and +/+ cranial NCC isolated at E10 and E11.5 (see Vogel and Parada, 1998, for method). We will compare proliferation on tissue plastic, fibronectin, and laminin for each genotype. Our preliminary results indicate that differences in proliferation within the 20-hr assay period do not account for the observed differences in invasiveness.

To compare invasiveness of neural crest-derived cells with other embryonic mesenchymal cells, we isolated fibroblasts from the trunks and limbs of E12 Nf1+/+, +/-, and -/- mouse embryos. **Figure 4** shows that no differences in invasiveness through fibronectin or laminin matrices were observed for

mouse embryo fibroblasts (MEFs) of the 3 different genotypes. Moreover, trunk MEFs were much less invasive than cranial NCC isolated at E10, E11, or E12 (compare **Figure 4** with **Figures 1,2,3**). MEFs isolated from E12 Nf1-/-;p53-/- mice and carried over 20 passages were still much less invasive than Nf1-/- NCC (data not shown). In fact, the motility of neurofibromin-deficient NCC through fibronectin and laminin matrices rivals that of our most invasive cisNf1;p53 sarcoma cell lines (**Figure 4**). Following completion of cranial NCC proliferation studies, and invasiveness assays with E10 and E12 trigeminal ganglion non-neuronal cells (in progress; anticipated completion of experiments by December 2002), we plan to submit the above data in a manuscript to the journal *Experimental Cell Research*. In the next year, we will analyze further the motile properties of Nf1-/- cranial NCC in two additional assays: 1) the Varani migration assay, and 2) back-transplantation to the neural crest migration pathway of an E2 chicken embryo. Both of these assays require very low cell numbers to obtain meaningful results, and thus we can include trunk NCC isolated from E9 mouse embryos in our studies. Milner and colleagues (1997) used the Varani assay, which measures the extent of cell migration from an agarose drop, to compare neonatal rodent Schwann cell motility on different extracellular matrix molecules. Not only can the dose-dependent effects of matrix molecules on migration be determined with these assays, but the roles of specific integrins in mediating motility, and the effects of growth factors such as TGFβ1 can be examined as well (Milner et al., 1997). We anticipate that these experiments would yield data sufficient for at least one additional manuscript. Finally, we have recently begun collaboration with UTHSCSA colleagues Drs. Sunil Ahuja and Yogesh Kalkonde, to examine the expression of chemokines and chemokine receptors in NC-derived cell populations. These signaling molecules modulate the motile behavior of lymphocytes and neuronal growth cones. Our preliminary data indicate that neurons and neuronal precursors in cultures of E11.5 trigeminal ganglia express the chemokine receptor CXCR4 (Y. Kalkonde, S. Ahuja, and K.Vogel, unpublished results). Following confirmation of these results, we plan to use the CXCR4 ligand (SDF-1) as a chemoattractant in transwell invasiveness assays with cranial NC-derived populations.

Task 3: Correlate integrin expression, cell adhesion, and responses to TGFβ1 with invasive and metastatic ability in mouse MTT sarcoma lines.

TGFβ1 and sarcoma cell invasiveness. Over the past year, we have concentrated our efforts on 1) correlating neural crest phenotype with invasiveness of cisNf1;p53 sarcoma lines and 2) characterizing the effects of TGFβ1 exposure on the invasiveness of cisNf1;p53 neural crest-derived sarcoma cells. First, we compared invasiveness of sarcoma lines that express different patterns of neural crest phenotypic traits. For example, Tu8-8 expresses early NCC markers (p75) and low levels of Schwann cell markers (S100), Tu9-6 expresses smooth muscle markers (calponin, alpha actin) and no Schwann cell markers, and Tu19-3 expresses neuronal markers (neurofilament, NeuN) and low levels of Schwann cell markers. All of the cell lines isolated from cisNf1;p53 mice exhibit loss-of-heterozygosity for both Nf1 and Trp53, and their invasiveness through fibronectin and laminin is compared in Figure 4. We have also included a sarcoma line, Tu24-4, that was isolated from a transNf1+/-;p53+/- mouse; Tu24-4 does not express any neural crest phenotypic markers, and it exhibits LOH at the Trp53, but not the Nf1, locus. The "leiomyosarcoma" cell line, Tu9-6, is not significantly more invasive than the non-neural crest cell line Tu24-4 (Figure 4). Another cisNf1;p53 sarcoma line, 61E9, that expresses high levels of smooth muscle markers fails to invade through either fibronectin or laminin matrices in these assays (data not shown). The "MPNST" line Tu8-8 is moderately invasive through fibronectin and highly invasive through laminin, whereas the neuronal sarcoma line Tu19-3 is highly invasive through both matrices (**Figure 4**). We propose to determine whether this correlation between NCC phenotype and invasiveness (neuronal>Schwann cell>smooth

muscle) exists in primary neural crest-derived cells isolated from Nf1+/+ and Nf1-/- mouse embryos.

We have continued to examine the effects of TGFβ1 exposure (3-5 days) on cisNf1;p53 sarcoma cell invasiveness in the transwell assays, and the results of duplicate experiments with TGFβ1 and TGFβ2 are summarized in **Figure 5**. Two cell lines, Tu8-8 and Tu26-4, showed minimal reduction in invasiveness through fibronectin, and no change in invasiveness through laminin, after 3-5 days of TGFb exposure. The leiomyosarcoma lines Tu9-4 and Tu9-6 showed a substantial decrease in invasiveness through fibronectin in response to TGFβ treatment; we know that expression of αV integrin mRNA is upregulated following TGFβ1 treatment in these lines (C. White and K. Vogel, unpublished results). The invasiveness of all neuronal sarcoma lines (Tu19-1, Tu19-3, and Tu19-7) through fibronectin and laminin matrices was significantly decreased by prolonged exposure to TGFβ (**Figure 5**). Finally, TGFβ treatment reduced invasiveness of the MPNST line Tu26-6; however Tu26-4, which was isolated from the same tumor, failed to respond. Our results were puzzling in light of the well-characterized effects of TGFβ on the invasiveness of glioma cells. For these highly migratory tumor cells, TGFβ *increases* invasiveness, and the effects on motility are apparent after brief exposure to the growth factor (Platten et al., 2000; Han et al., 2001; Wick et al., 2001). Therefore, we examined the effects of 24-hour TGFβ exposure on cisNf1;p53 sarcoma cell invasiveness. **Figure 6** compares the effects of different periods of TGFβ1 exposure on invasiveness through fibronectin and laminin for the neuronal cell line Tu19-7. Short-term (24-hr) exposure to TGFβ1 actually increases the invasiveness of Tu19-7 sarcoma cells, just as it does for glioma cells.

TGFβ and neural crest differentiation. TGFβ1 has a variety of effects on embryonic and postnatal neural crest derivatives. Rat embryo neural crest stem cells respond to TGFβ1 treatment by undergoing smooth muscle differentiation (Shah et al., 1996). However, when these neural crest stem cells are cultured as "communities", rather than in isolation, low doses of TGFβ1 promote differentiation along the autonomic neuron lineage, and higher doses of TGFβ1 induce apoptosis (Hagedorn et al., 2000). Neural crest-derived Schwann cells produce and secrete TGFβ, which may regulate cell number through induction apoptosis during development, and following nerve injury (Parkinson et al., 2001). In several of our cisNf1;p53 sarcoma lines, TGFβ1 treatment over a period of 3-5 days increases the level of expression of the smooth muscle markers alpha actin and calponin. This summer we were able to quantitate the effects of TGFβ1 and basic fibroblast growth factor (FGF) exposure on the expression of neuronal markers in our sarcoma lines Tu19-1, Tu19-3, Tu19-4, and Tu19-7. **Figure 7** shows that prolonged (3-5 days) exposure to TGFβ1 decreases the expression of neurofilament protein in these sarcoma lines; in contrast, bFGF treatment increases expression of this neuronal marker. TGFβ1 signaling can involve a combination of several different intracellular signaling pathways, and we propose to use inhibitors to determine which pathways are involved in short-term and long-term effects on invasiveness, and in the modulation of neural crest phenotypes in the cisNf1;p53 sarcoma lines. Because the effects of TGFβ1 on the neuronal sarcoma cells Tu19-1, Tu19-3, Tu19-4, and Tu19-7, and on the leiomyosarcoma lines Tu9-4 and Tu9-6 are particularly dramatic and reproducible, we will limit our signal transduction studies to these lines. In conjunction with the experiments on neural crest phenotype, we quantitated proliferation of cisNf1;p53 sarcoma lines in response to TGFβ1, TGFβ2, bFGF, or IGF1 treatment, using an immunocytochemical BrdU assay. The proliferation experiments were completed in my laboratory by Candace Chan, a 2002 Summer Undergraduate Research Fellowship student; the SURF program at UTHSCSA provides stipends for Texas college students to undertake research projects in funded laboratories. Sarcoma cells were maintained in 2% HIFCS/DMEM for 3 days in the presence of growth factors, and then given a 12-hour pulse of BrdU. Ms. Chan's data are summarized in **Figure 8**, and show that the

cisNf1;p53 sarcoma lines Tu8-8, Tu9-6, and Tu19-10 respond differentially. All three lines respond to bFGF with increased proliferation; however, Tu19-10 does not exhibit a significant response to TGFβ.

We propose the following series of experiments and strategies to facilitate preparation of a manuscript: 1) Focus on a maximum of 6 different cisNf1;p53 sarcoma lines 2) Address the issue of the temporal effects of TGFβ exposure through use of pharmacological inhibitors of different TGFβ signaling pathways 3) Determine whether TGFβ signaling effects on sarcoma cell invasiveness and differentiation utilize the same intracellular pathways 4) Examine levels of TGFβ receptor levels in the 6 sarcoma lines.

Task 4: Analyze role of Ras signaling in locomotory, invasive, and metastatic properties of mouse MTT sarcoma lines.

To complete the initial set of experiments outlined in this task, we will use an assay based on the unique ability of GTP-bound (activated) Ras to bind to the downstream signaling molecule Raf. Our first goal will be to compare activated Ras levels between cisNf1;p53 sarcoma cell lines that exhibit different invasiveness properties. To facilitate completion of these experiments within the next year, we have enlisted the support of Dr. Lily Dong, who joined the Cellular and Structural Biology department this summer. Dr. Dong has extensive experience with the Raf pull-down assay, and will provide us with some of the reagents, as well as technical advice (letter provided in Appendices section).

Figure Legends

Figure 1. Neurofibromin-deficient E10 mandibular arch mesenchymal cells are more invasive through laminin. 5000 mesenchymal cells were plated on top of a fibronectin (10 μg/ml) or laminin (10μg/ml) matrix in each Costar transwell, and allowed to migrate for 18-20 hours at 37C. The chemoattractant was 2% fetal calf serum, and cells were fixed in methanol and stained with 1% crystal violet. 15 fields were counted for each transwell at a magnification of 200X. Each bar represents the average of results obtained from at least three different embryos from 3 different litters, with 2-3 transwell membranes counted for each embryo per matrix molecule. Error bars represent ranges of values obtained.

Figure 2. Neurofibromin-deficient E11.5 first arch mesenchymal cells are more invasive through fibronectin and laminin. Mesenchymal cells were plated in transwells as above, and allowed to migrate for 18-20 hours. Each bar represents the average of results obtained from at least three different embryos from 2 different litters, with 3 transwell membranes counted for each embryo per matrix molecule. Error bars represent ranges of values obtained.

Figure 3. Neurofibromin-deficient E11.5 trigeminal cells are more invasive through fibronectin and laminin. Non-neuronal cells from trigeminal ganglia were plated in transwells as above, and allowed to migrate for 18-20 hours. Each bar represents the average of results obtained from 2-3 different embryos, with 3 transwell membranes counted for each embryo per matrix molecule. Error bars represent ranges of values obtained.

Figure 4. Comparison of MEF, transNf1;p53 sarcoma, and cisNf1;p53 sarcoma invasiveness through fibronectin and laminin. For all cell types, 5000 cells were plated on top of the matrix in each transwell, and cells were allowed to migrate for 18-20 hours. For each bar, at least 6 transwells were counted; error bars represent ranges of values obtained.

Figure 5. TGFβ1 treatment reduces invasiveness for most cisNf1;p53 sarcoma lines. Each bar represents data combined from 2 different experiments, with a total of 6 transwells counted for each condition.

Figure 6. Temporal effects of TGFβ1 exposure on the invasiveness of cisNf1;p53 sarcoma line Tu19-7. Sarcoma cells were exposed to TGFβ1 (5 ng/ml) for 1, 3, or 5 days prior to transwell invasiveness assay. Each bar represents data combined from 2 different experiments, with a total of 6 transwells counted for each condition. Error bars represent ranges of values obtained.

Figure 7. TGFβ1 treatment decreases expression of neurofilament protein, whereas bFGF increases neuronal differentiation in cisNf1;p53 sarcoma lines. Tumor cells plated on coverslips were exposed to TGFβ1 (5 ng/ml) or bFGF (10 ng/ml) for 3-4 days, and then immunostained with an anti-neurofilament160 antibody.

Figure 8. Differential proliferative response of cisNf1;p53 sarcoma lines to growth factors. Tumor cells plated on coverslips were exposed to growth factors for 3 days, and then incubated with BrdU for 12 hours. Cells were fixed and immunostained with an anti-BrdU antibody. Total nuclei were counted following labeling of DNA with Hoechst 33258.

Figure 1.
Invasiveness of E10 Mandibular Arch Mesenchymal Cells

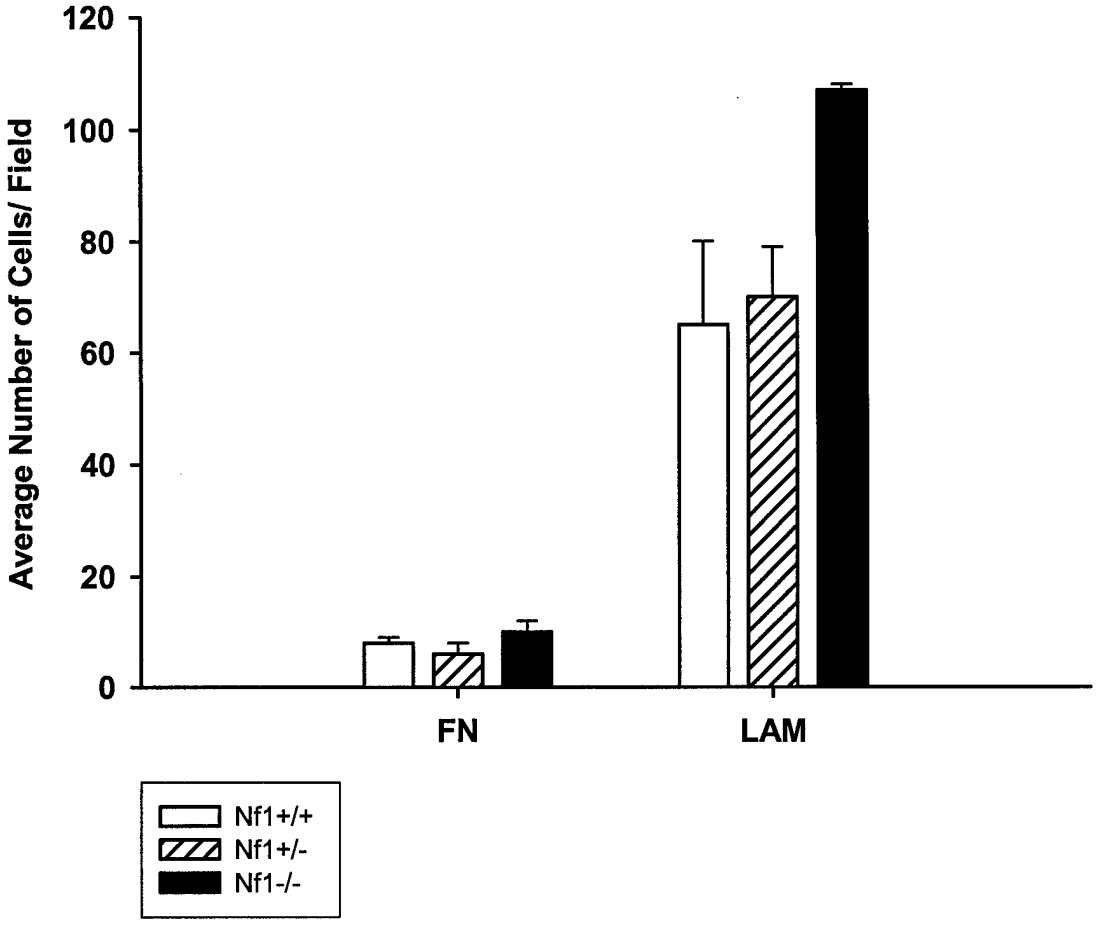


Figure 2.
Invasiveness of E11.5 Maxillary and
Mandibular Arch Mesenchymal Cells

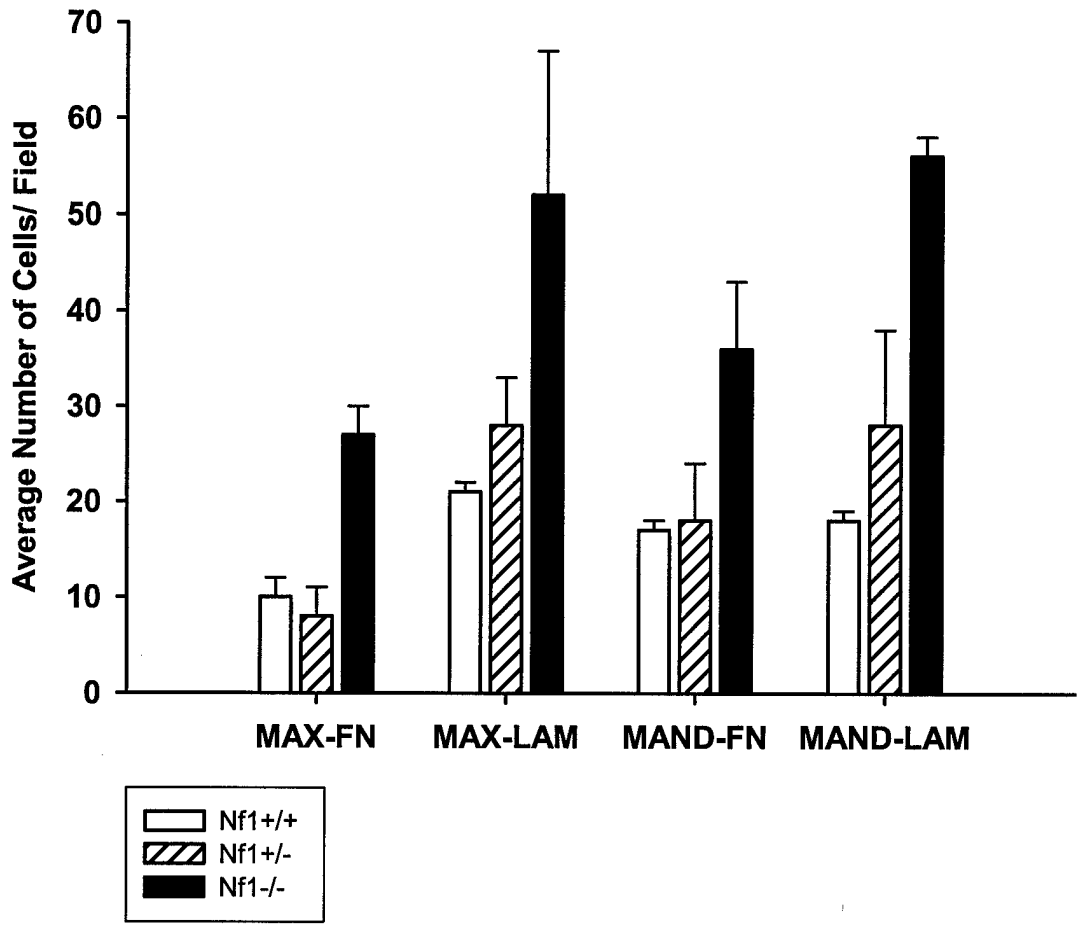


Figure 3.
Invasiveness of E11.5 Trigeminal Ganglion
Non-neuronal Cells

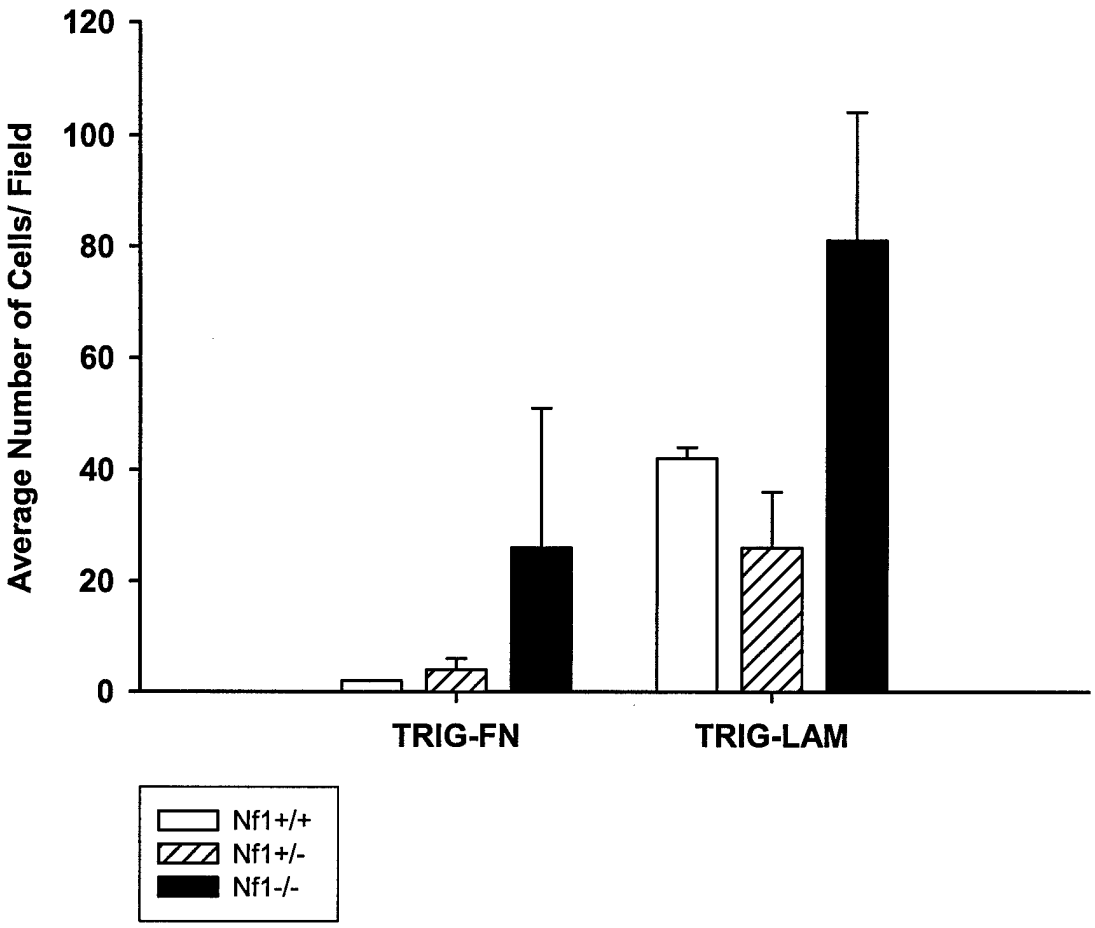


Figure 4.
Invasiveness Through Fibronectin and Laminin:
E12 MEFs, trans and cisNf1;p53 Sarcoma Cells

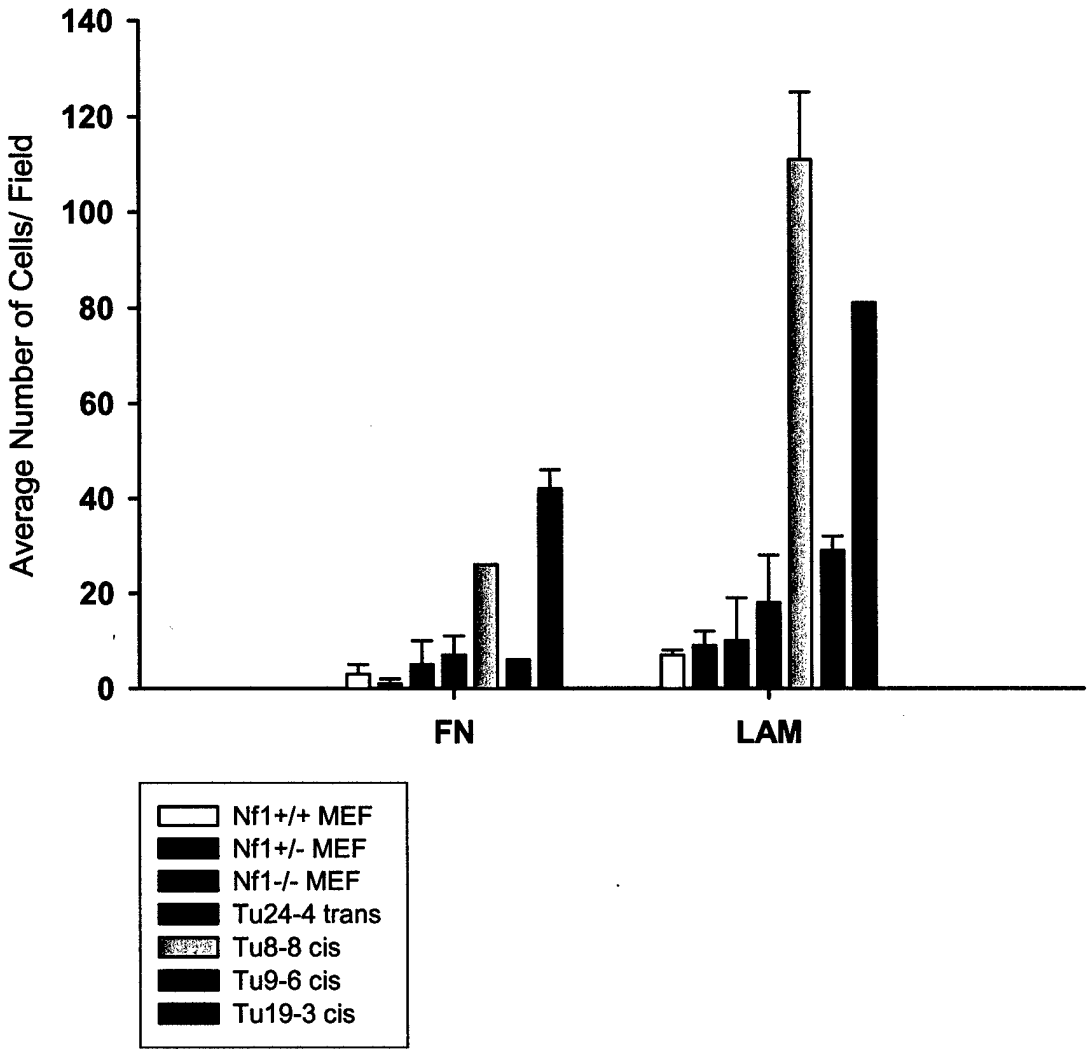


Figure 5.
Effect of TGFβ1 Treatment on Invasiveness
of cisNf1;p53 Sarcoma Cell Lines

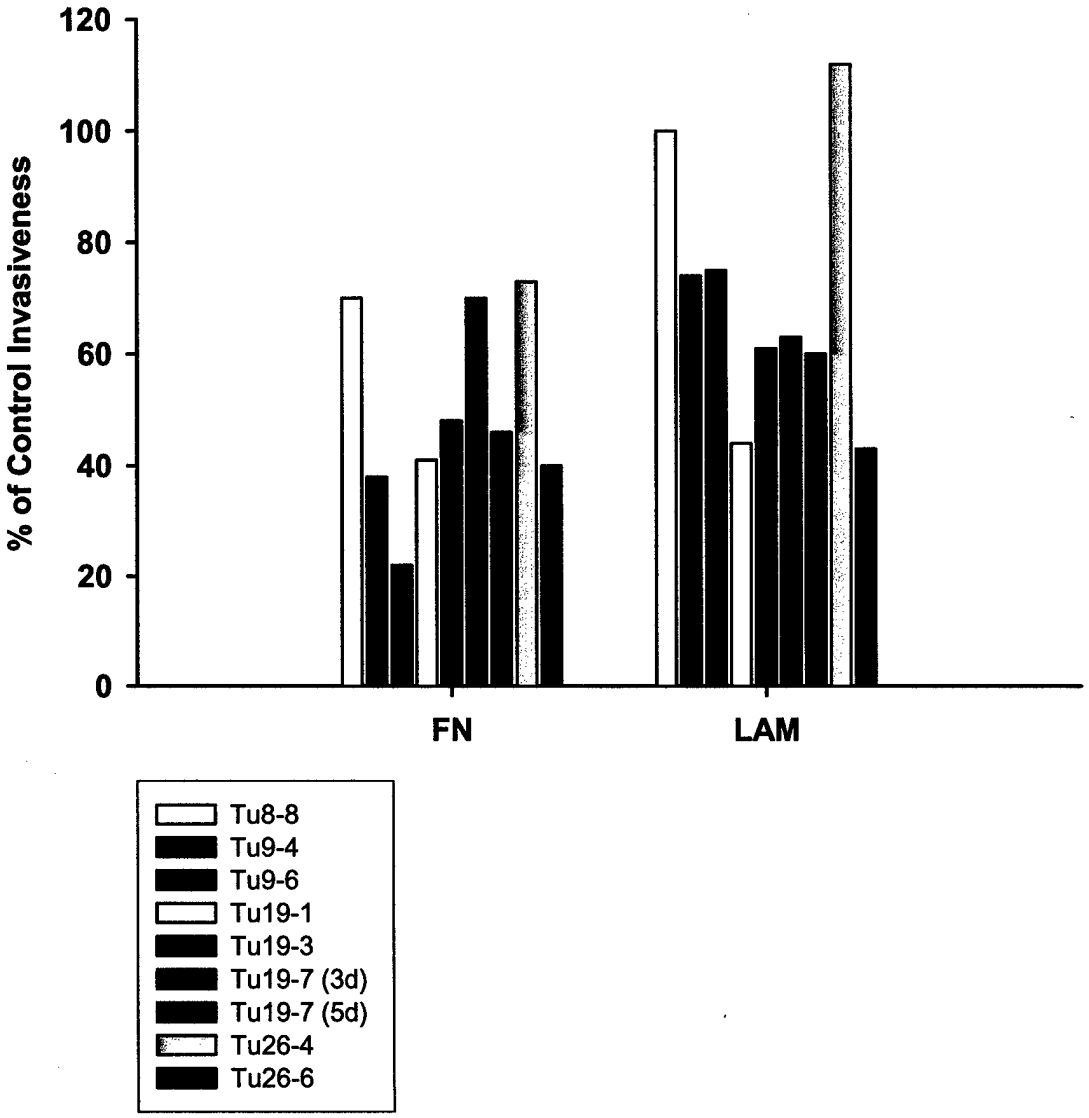


Figure 6.
Effects of TGFβ1 Exposure on Invasiveness
cisNf1;p53 Sarcoma Cell Line Tu19-7

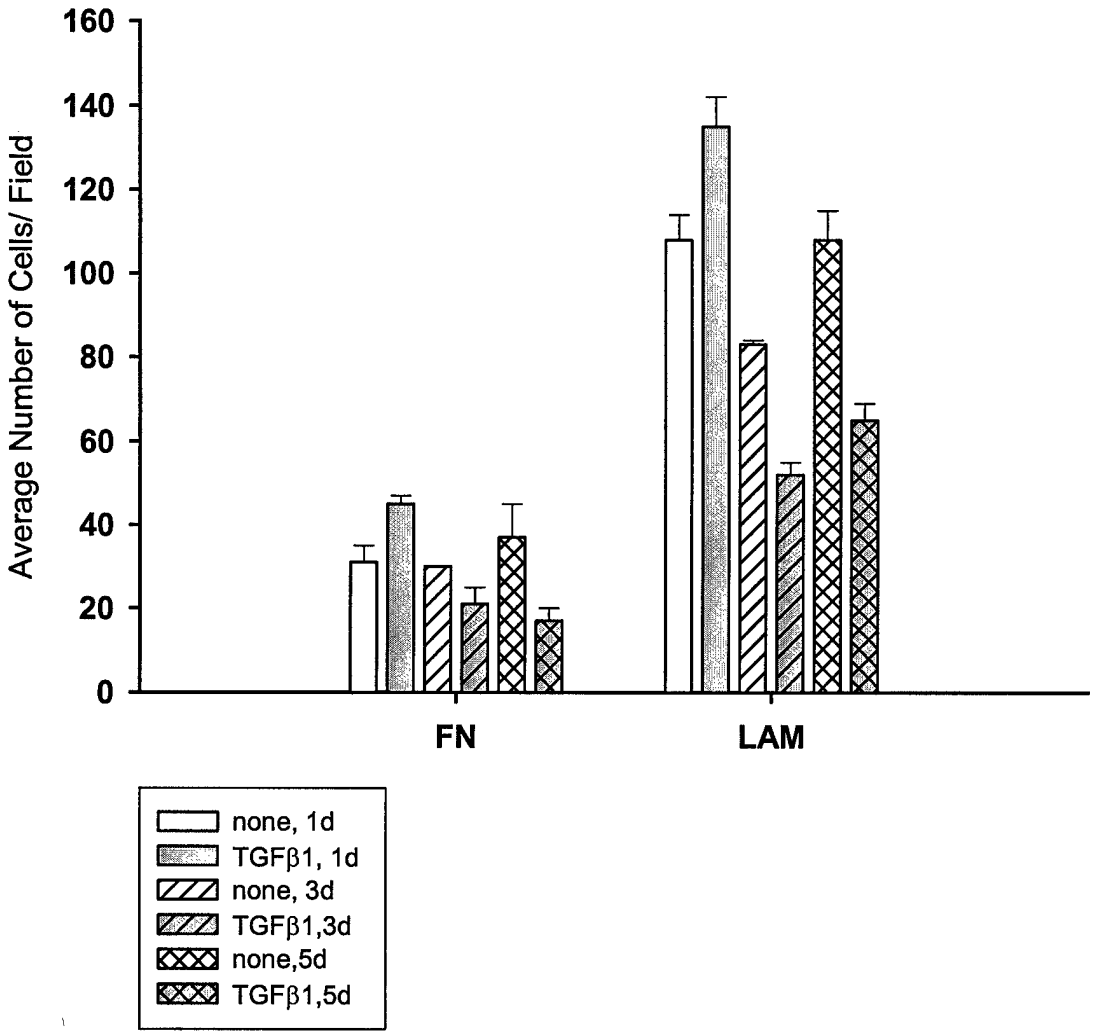


Figure 7.
Effects of bFGF and TGFβ1 on Expression of Neuronal Markers

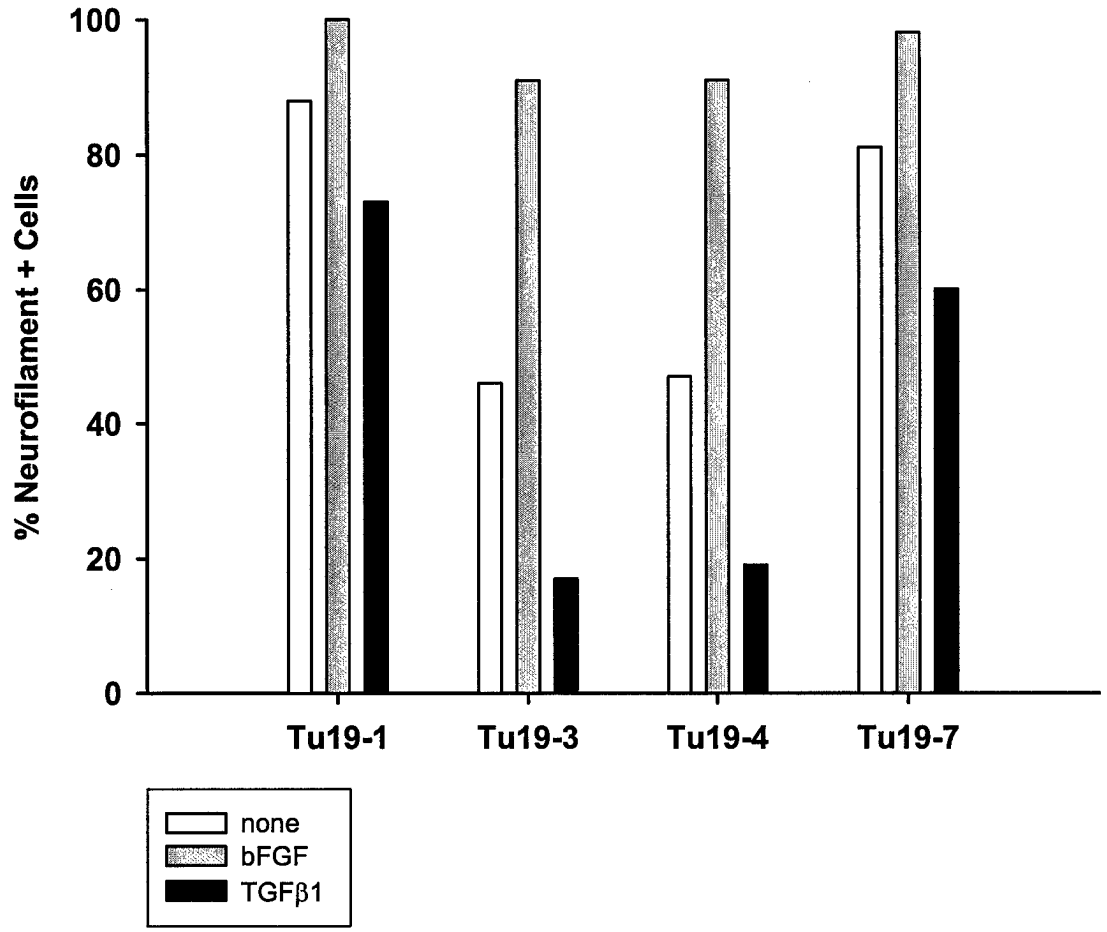
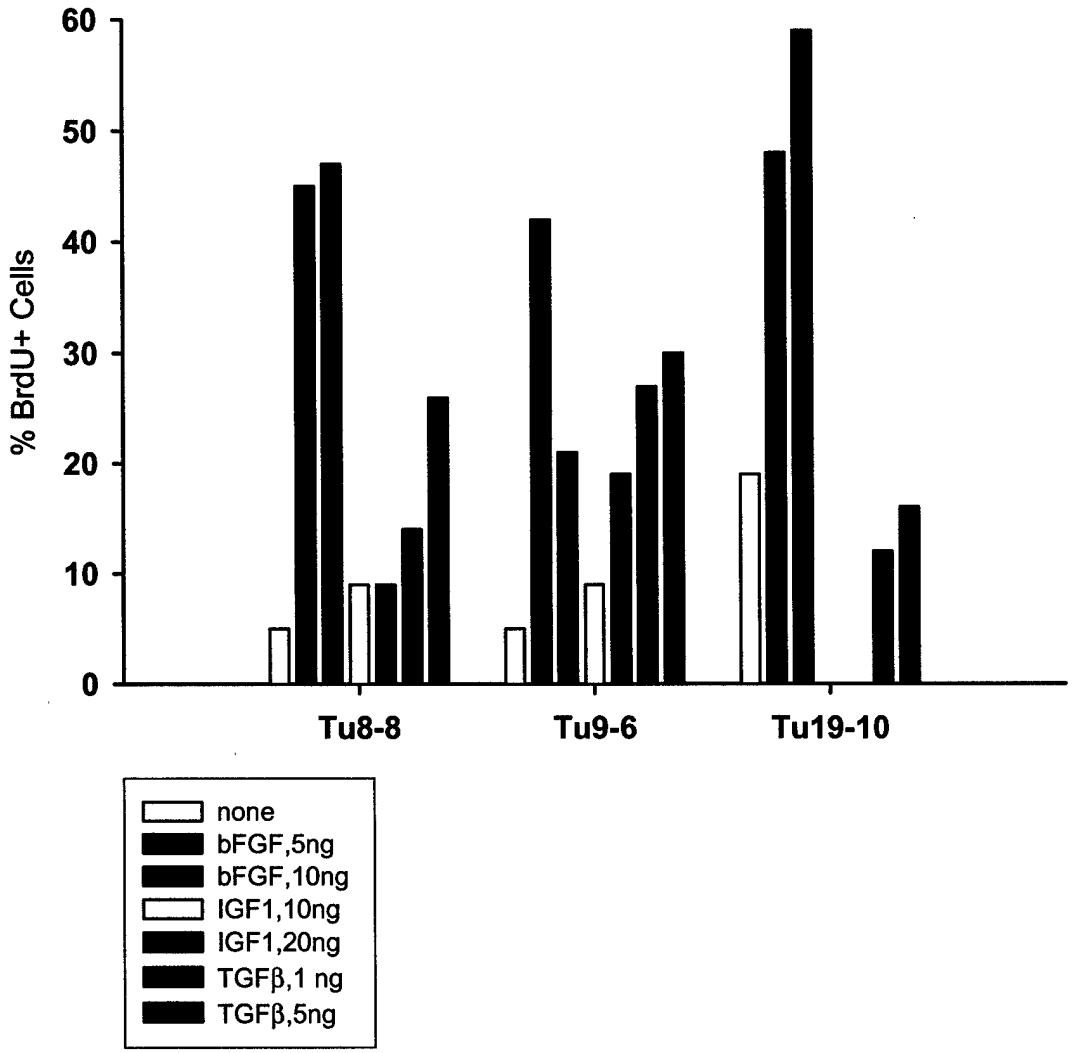


Figure 8.
Effects of Growth Factors on cisNf1;p53
Sarcoma Cell Proliferation



KEY RESEARCH ACCOMPLISHMENTS

- Completed experiments on invasiveness of E10-E11.5 maxillary and mandibular arch mesenchymal cells isolated from Nf1+/+, +/-, and -/- mouse embryos
- Developed techniques for isolation and expansion of neural crest cell populations from embryonic trigeminal ganglia
- Completed experiments on invasiveness of E11 trigeminal non-neuronal cells through fibronectin and laminin (Nf1+/+, +/-, -/-); experiments on E10 and E12 trigeminal cells in progress
- Completed experiments to correlate cisNf1;p53 sarcoma cell phenotype with invasiveness through fibronectin and laminin matrices
- Completed experiments on effects of TGFβ1 exposure on the invasiveness of cisNf1;p53 sarcoma lines; identified critical time period of exposure for reduction in invasiveness
- Examined effects of bFGF and TGFβ on cisNf1;p53 sarcoma cell proliferation

REPORTABLE OUTCOMES

Presentations

January 2002: Invited seminar, Western Michigan University, Biology Department. "Neurofibromin: A signaling molecule with roles in neuronal survival and tumorigenesis"

June 2002: Molecular Biology of NF1 and NF2 Meeting, Co-chair session on Tumorigenesis. "Invasiveness of neurofibromin-deficient neural crest cells and sarcoma cell lines" (talk)

Abstracts

T. Starr, C. White, and K.S. Vogel (2002). Invasiveness of neurofibromin-deficient neural crest cells and sarcoma cell lines. Presented at the 2002 Molecular Biology of NF1 and NF2 Meeting. (included in Appendices section)

Training

- **Christopher White, M.D.** Dr. White is a General Surgery resident with a background in RNA isolation and RT-PCR. He has contributed to the sarcoma cell invasiveness assays, and is analyzing the effects of TGFβ exposure on integrin mRNA expression.
- **Candace Chan** Ms. Chan was a Summer Undergraduate Research Fellow in my laboratory, and examined the effects of growth factors on cisNf1;p53 sarcoma cell proliferation. She also contributed to the studies on neurofilament protein expression.

Animal Models

Crossed cisNf1+/-;p53+/- mice with Stratagene Big Blue (lacI transgenic) mice, to measure mutant frequency in neural crest-derived sarcomas, and to determine whether a correlation exists between neural crest phenotype and mutant frequency.

Funding

Applied for funding from the San Antonio Area Foundation to support pilot project on mutant frequency in cisNf1;p53 sarcomas.

CONCLUSIONS

In a variety of different cell types, haploinsufficiency for or loss of Nf1 function results in increased invasiveness. Both Nf1+/- and Nf1-/- Schwann cells exhibit increased invasiveness through Matrigel in transwell assays (Kim et al., 1997). Similarly, Nf1+/- and Nf1-/- astrocytes display decreased cell attachment, actin cytoskeletal abnormalities, and increased invasiveness through Matrigel (Gutmann et al., 2001). For branchial arch mesenchymal cells and trigeminal neural crest cells, we have shown that loss of neurofibromin results in increased invasiveness through fibronectin and laminin. Identification

of the intracellular signal transduction pathways involved in this altered motility is a priority; Rangwala and colleagues (2002) recently reported that both R-Ras and PI3 kinase may mediate neurofibromin-dependent changes in migratory potential. In contrast, neurofibromin does not appear to play a role in melanocyte precursor dispersal on the lateral pathway *in vivo* (Wehrle-Haller et al., 2001). Therefore, to characterize changes in neural crest cell motility that result from neurofibromin deficiency, a combination of *in vitro* (transwell assays, Varani migration assays) and *in vivo* (back-transplant to NCC migration pathway) techniques will be used.

By altering the duration of TGFβ treatment, we have shown that neural crest-derived sarcoma cells respond to brief exposure with an increase in invasive potential, and to prolonged exposures with a decrease in migratory ability. This change in invasiveness is correlated with alterations in sarcoma phenotype, but we have yet to identify a direct link. We will continue to analyze integrin expression in these cell lines, with particular attention to αV integrin, which is up-regulated in malignant glioma cells in response to TGFβ treatment (Platten et al., 2000). In addition, we plan to focus our investigations on signal transduction pathways induced by TGFβ treatment, with a particular emphasis on Ras activation, in selected cisNf1;p53 sarcoma lines. Our experiments address important questions in the areas of neural crest cell migration during embryonic development and motility of neural crest-derived sarcoma cells in the adult. Both cell populations are likely to use similar combinations of integrin ECM receptors and intracellular signaling pathways during migration, and results obtained in one system will facilitate understanding of the other.

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ABSTRACT FORM (Abstract not to exceed this frame)

TITLE :___Invasiveness of neurofibromin-deficient neural crest cells and sarcoma cell lines
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Many of the cell types affected in neurofibromatosis type 1 are derived from the neural crest, a population of embryonic cells that migrate extensively and localize to differentiate as sensory and sympathetic neurons, Schwann cells, melanocytes, and skeletal derivatives of the branchial arches. The product of the Nf1 gene, neurofibromin, is a GTPase-activating protein (GAP) that negatively regulates growth factor signaling through Ras in several different neural crest derivatives. Neurofibromin may also interact with microtubules. We have examined the motile properties of neural crest-derived cells isolated from Nf1-/- mouse embryos in two different assays. First, we assessed the ability of sensory (trigeminal and dorsal root ganglion) neurons to extend axons into appropriate (whisker pad or forepaw) or inappropriate (heart) targets in an explant coculture system. Although Nf1-/- DRG and trigeminal neurons extend axons with normal patterning into whisker pad or forepaw, they aberrantly extend axons into heart explants. DRG or trigeminal neurons isolated from wild-type littermates never extend axons into heart explants. Second, we compared the invasiveness of branchial arch neural crest cells isolated from E10 and E11 Nf1-/-, Nf1+/-, and Nf1+/+ mouse embryos through fibronectin and laminin matrices in a transwell assay. We found that mandibular and maxillary arch neural crest cells isolated from Nf1-/- embryos were more invasive through fibronectin and laminin than those isolated from Nf1+/- and Nf1+/+ littermates.

Although Nf1+/- mice do not develop neurofibromas, mice harboring targeted null mutations in Nf1 and Trp53 in cis spontaneously develop neural crest-derived soft tissue sarcomas, similar to malignancies that appear in NF1 patients. To characterize the motile and invasive properties of neurofibromin-deficient cells, we have developed clonal tumor cell lines from soft tissue sarcomas that arise in cisNf1+/-;p53+/- mice. Neural crest-derived sarcoma lines exhibit differential expression of Schwann cell, smooth muscle, and neuronal traits, and respond to glial growth factor and transforming growth factor beta 1 (TGFβ1) in a manner similar to normal embryonic neural crest stem cells. TGFβ1 can modify cell motility by altering the expression patterns of integrin extracellular matrix receptors. To characterize the effects of this growth factor on the invasive properties of neural crest-derived sarcoma cells, we compared invasiveness through fibronectin and laminin matrices for TGFβ1-treated and untreated cultures. For sarcoma cell lines that express either Schwann cell or neuronal traits, we found that TGFβ1 treatment for 3-4 days significantly reduces invasiveness through fibronectin and laminin matrices in transwell assays. We are currently characterizing patterns of integrin expression in these cell lines, and changes in expression following TGFβ1 treatment.

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Dear Kris:

I have read your grant entitled "Cell Motility and Invasiveness of Neurofibromin-deficient Neural Crest Cells and Malignant Triton Tumor Lines" with a great interest. As you know, I have been working on insulin/IGF-1 signal transduction and regulation for the past 7 years and I have successfully used the GST-Raf-RBD pull-down assay to study insulin-stimulated Ras activation (Wick et al. (2001) J. Biol. Chem., 276, 42843-42850). I would be most pleased to assist you with the Raf binding assay as proposed in your study and I look forward to working with you on this very interesting and important project. Please feel free to contact me if you have any questions.

Best wishes,

Lily Dong, Ph.D.
Assistant professor